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Regulation of myosin light chain 2 phosphorylation in cardiac muscle and its possible role in contractile responses

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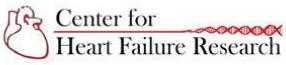

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Hilde Eikemo
Trondheim, April 2009

DEFINITIONS/ABBREVIATIONS

BIM = bisindolylmaleimide

caly A = calyculin A

CaMKII = Ca^{2+}/calmodulin-dependent kinase II

CM = cardiomyocyte

cMLCK = cardiac myosin light chain kinase

DMSO = dimethyl sulfoxide

EGTA = ethylene glycol tetraacetic acid

GPCR = G-protein-coupled receptor

MHC = myosin heavy chain

MLC-1 = myosin light chain 1/essential light chain (ELC)

MLC-2 = myosin light chain 2/regulatory light chain (RLC)

MLC-2-P = phosphorylated myosin light chain 2

MLCK = myosin light chain kinase

MLCP = myosin light chain phosphatase

PKC = protein kinase C

ROCK = Rho kinase

skMLCK = skeletal muscle MLCK

smMLCK = smooth muscle MLCK

stsp = staurosporine

LIST OF PAPERS

Paper I:

H Eikemo, T Skomedal, FO Levy, J-B Osnes. Calculating reaction rate constants and estimating the efficacy of selective enzyme inhibitors. Anal Biochem 2008; 383: 323-325

Paper II:

H Eikemo, CHT Nguyen, FO Levy, T Skomedal, J-B Osnes. CaMKII and at least two unidentified kinases phosphorylate myosin light chain 2 in rat quiescent cardiomyocytes. *Submitted*

Paper III:

H Eikemo, RI Hussain, CHT Nguyen, FO Levy, T Skomedal, J-B Osnes. Electrical stimulation of adult cardiomyocytes recruits MLCK activity *in situ. Manuscript*

Paper IV:

RI Hussain, E Qvigstad, JAK Birkeland, H Eikemo, A Glende, I Sjaastad, T Skomedal, J-B Osnes, FO Levy, KA Krobert. Muscarinic receptor activation elicits an inotropic effect in failing rat ventricle through myosin light chain phosphorylation. Br J Pharmacol 2009; 156:575-86

INTRODUCTION

Regulation of cardiac function in terms of contractility, beating frequency, blood supply and metabolic rates, relies on a three-tiered control system: i) immediate and fast feedback in response to mechanical load on a beat-to-beat basis (Frank-Starling relation), ii) more sustained regulation involving transmitters and hormones as primary messengers and iii) long-term adaptation by changes in the gene expression profile. The more sustained signalling mode involves a wide variety of biologically active molecules mediating their effects through receptors in the heart. Signal transduction pathways link the events on the cell surface with various effector mechanisms within the cardiomyocyte, including modulation of the contractile apparatus [1]. This thesis aims at elucidating aspects of how the phosphorylation of the small protein myosin light chain 2 (MLC-2) is regulated, and its possible role in modulating contraction of the heart.

Cardiac muscle; anatomy and physiology

Cardiac muscle is a type of muscle tissue found only in the walls of the heart. It is similar to skeletal muscle with respect to being striated and similar to smooth muscle with respect to the nuclei being centrally located and many cells are required to span the length of the muscle. It differs from both skeletal muscle and smooth muscle in that its cells branch and are joined to one another via intercalated disks; densely staining transverse bands that characteristically appear at right angles to the long axis of the cardiac myofibers, and that represent specialized cell-cell junctions that contain regions of low electrical resistance. This allows communication between the cells producing a sequential contraction of the cells from the bottom of the ventricle to the top. Although not a true anatomical syncytium, the heart functions as if all of the cardiomyocytes are in free electrical communication [2].

Cardiomyocytes

Although cardiomyocytes represent most of the myocardial mass, approximately 70% of the cells are smaller nonmyocytes including vascular smooth muscle cells, endothelial cells and fibroblasts. Also, there are several types of cardiomyocytes within the adult human heart. The most numerous are *working cardiomyocytes* of the atria and ventricles that are specialized for contraction. Atrial cardiomyocytes are smaller in diameter than those of the ventricles. *Purkinje fibers*, found in the AV bundle, bundle branch and ventricular endocardium, are large cells specialized for rapid conduction. The SA and AV node are networks of small, sparsely cross-striated *nodal cells* [2].

The sarcomere

The contractile proteins, which make up almost 50% of the volume of working cardiomyocytes, are organized in a regular array of cross-striated myofibrils. The fundamental morphological unit of striated muscle is the sarcomere, defined as the region between two Z lines (figure 1).

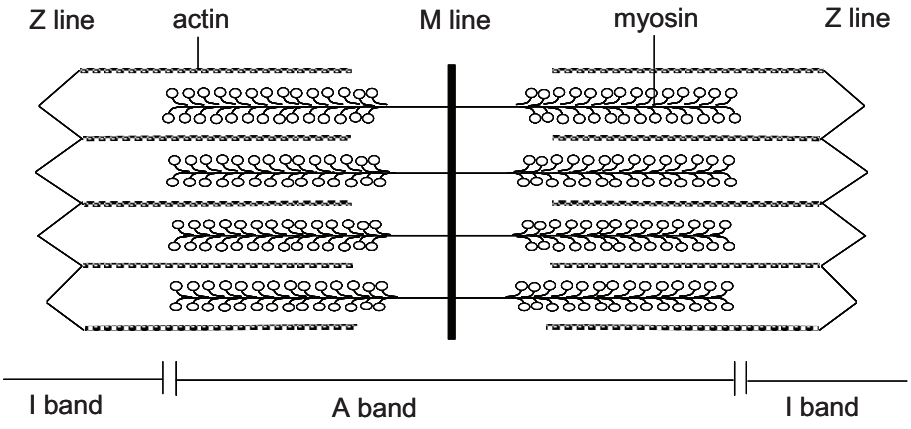

Figure 1*: Simplistic model of a sarcomere.*

The thick filaments that extend the length of the A-band are polymers of myosin and titin. The central regions of the thick filaments also contain myosin-binding protein C, M-protein, myomesin and an isoform of creatine phosphokinase. The thin filaments are double-stranded actin polymers that include tropomyosin and the three proteins of the troponin complex; troponin T, C and I. At the Z lines, the thin filaments are interwoven with several cytoskeletal proteins, including α-actinin, Cap Z (β-actinin), nebulette and desmin, which attach the sarcomeres to cell adhesion molecules that link myocytes to each other and to the extracellular matrix [2].

Myosins are motor proteins known to play fundamental roles in many forms of eukaryotic motility such as cell crawling, cytokineses, phagocytosis, maintenance of cell shape, and organelle/particle trafficking [3]. Although actin polymerization alone can drive some forms of motility, myosins appear to power a wide variety of movements and are important in processes such as signal transduction [4] and establishment of polarity [5].

Members of the myosin superfamily are defined by the presence of a heavy chain with a conserved catalytic domain. In most myosins, the catalytic domain is followed by an α-helical light chain-binding region consisting of one or more IQ motifs, and a C- and/or an N-terminal extension thought to endow class-specific properties such as membrane binding or kinase activity [3].

In the human heart, two sarcomeric myosin II genes are expressed, generating two isoenzymes of myosin heavy chain (MHC) (designated α- and β-MHC), which share 93% structural homology in the rat [6]. The relative proportion of α- and β-MHC depends on species, age, hormonal balance and cardiovascular stress [7-11]. More specifically, small mammals such as adult rodents (e.g. mouse and rat) predominantly express α-MHC in the ventricle, whereas larger mammals (e.g. rabbits and humans) predominantly express β-MHC [12]. In rodents, β-MHC reveals a lower ATPase activity than α-MHC and is associated with a lower maximal shortening velocity of the cardiac fibers [13, 14].

Human cardiac muscle myosin is a hexameric protein of 520 kDa, consisting of two heavy chains (MHC, ~220 kDa) and two pairs of light chains (MLC): the essential light chain (MLC-1/ELC, alkali MLC, ~20 kDa) and the regulatory light chain (MLC-2/RLC, 5,5-dithio-bis-(2-nitro-benzoate)-MLC, ~22 kDa). Each MLC is bound to an α-

helix (lever arm) that links the globular N-terminal head domain (~120 kDa) to the rod portion (~100 kDa). Beneath the lever arm the two MHC molecules, together with the associated light chains, form an α-helical coiled-coil that extends into the thick filament backbone. There, double-stranded myosin molecules anneal with adjacent double-stranded myosin molecules, entering the stack at 14.3 nm intervals, 3 pairs of heads, arrayed at 120° intervals in cross-section. A pair of myosin heads is stationed every 42.9 nm, up to the mid point (M-line), where the polarity of the myosin reverses so that the summed forces of the actomyosin cross-bridges in the thick filament serve to shorten the sarcomere [15].

The actomyosin motor

The actomyosin motor consists of two parts: the head region of myosin that extend from the thick filament surface, and actin monomers of adjacent thin filaments, to which the myosin heads bind during force generation and muscle shortening. The motor is fueled by MgATP, and regulated by Ca^{2+} which has evolved as a global intracellular messenger for signal transduction by reversible binding to Ca^{2+}-sensing proteins. Inside the cardiomyocyte, ion pumps, ion exchangers and channels keep the cytoplasmic calcium level at rest around ~100 nM which is more than 10,000-fold lower than outside the cell ($>10^{-3}$ M). Upon cell membrane depolarization, the cytoplasmic Ca^{2+} concentration increases about 20-fold to above 1-10 μM, happening in the range of milliseconds [1, 16].

Upon depolarization of the transverse tubular membrane (invaginations of the sarcolemma at the Z lines), there is influx of Ca^{2+} through the voltage sensitive slow inward Ca^{2+}-channel that triggers Ca^{2+} release from the ryanodine receptor (Ca^{2+}-release channel of the sarcoplasmic reticulum (SR)). Ca^{2+} activates the troponin-tropomyosin complex on actin-containing thin filament, thereby opening target sites on actin monomers to myosin head binding. The myosin head contains a crucial nucleotide-binding site to which MgATP binds. The enzymatic activity of myosin helps water split the terminal phosphate from the ATP by breaking the covalent bond. The products of $MgATP^{2-}$, $MgADP^{1-}$ and P_i^{2-}, remains bound, and the myosin head binds to actin via weak ionic interactions at specific residues at the myosin-actin interface. Ca^{2+}-regulated movement of tropomyosin allows hydrophobic interactions to occur between myosin and

actin, thereby creating a much tighter bond [15, 17, 18]. When myosin binds to actin, an ATP-dependent conformational change in the myosin molecule results in movement of the actin filaments towards the centre of the sarcomere. This movement, also known as the cross-bridge cycle, shortens the length of the sarcomere and leads to contraction of the muscle fibre.

Recently, an interesting discovery was made concerning the anatomy of the cardiomyocytes. Wu and Bers [19] demonstrated that the SR lumen is highly interconnected with the nuclear envelope and also with distant regions of the SR. Moreover, the SR-nuclear envelope throughout the cardiomyocyte appears to be a single large continuous Ca^{2+} storage compartment. This provides a spatially uniform driving force for SR Ca^{2+} release throughout the cardiomyocyte during depolarization, and may be important in producing a homogeneous and synchronous Ca^{2+} release and contractile activation. Additionally, it might connect cytoplasmic and nuclear events, e.g. contraction and gene expression.

Myosin light chain

In the human heart, two different genes encode MLC-1 isoforms; an atrium-specific and a ventricle-specific. Ventricular MLC-1 is the same isoform as the MLC-1 present in the adult slow skeletal muscle [20]. MLC-2 also exists in an atrial and ventricular isoform, but in contrast to MLC-1, MLC-2 can be phosphorylated. Furthermore, sequence analyses in humans and various animal species suggest the existence of two ventricular MLC-2 isoforms (MLC-2 and MLC-2*) having the same molecular weight but different isoelectric points (MLC-2* being more acidic) [20, 21]. MLC-2* is the dominant isoform expressed in humans (ratio 2.3 to MLC-2) [22]. Adult rat and mouse appear to be an exception having only two isoforms (MLC-1 and MLC-2) [23]. Together with calmodulin and troponin C, the myosin light chains belong to the superfamily of EF-hand Ca^{2+} binding proteins [24]. Post-translational modifications other than phosphorylation have also been identified in the functionally important N-terminal site of MLC-2. In normal hearts, both phosphorylation and deamidation (the latter is thought to account for the acidic shift in MLC-2*) have been documented, whereas N-terminal truncation has been associated with ischemia/reperfusion [25].

All striated muscles make use of molecular mechanisms that help positioning the myosin heads for optimal force and power output. Studies in animals have revealed that a key determinant is the surface-to-surface distance between thick and thin filaments [26-28]. Phosphorylation of MLC-2 at Ser^{15} results in the addition of negative charge to the N-terminal region of MLC-2. This causes the myosin heads to swing out from the thick filament backbone towards the actin filament (the "order to disorder" transition), thereby increasing the probability of attachment to actin and promoting force generation (figure 2) [29, 30]. This fine-tuned steric translocation of the myosin head is the basis for Ca^{2+} sensitisation. MLC-2 phosphorylation-induced Ca^{2+} sensitization has no effect upon steady-state isometric force at saturating concentrations of Ca^{2+}, but only at concentrations necessary to elicit ~60% of maximum isometric force [31-33].

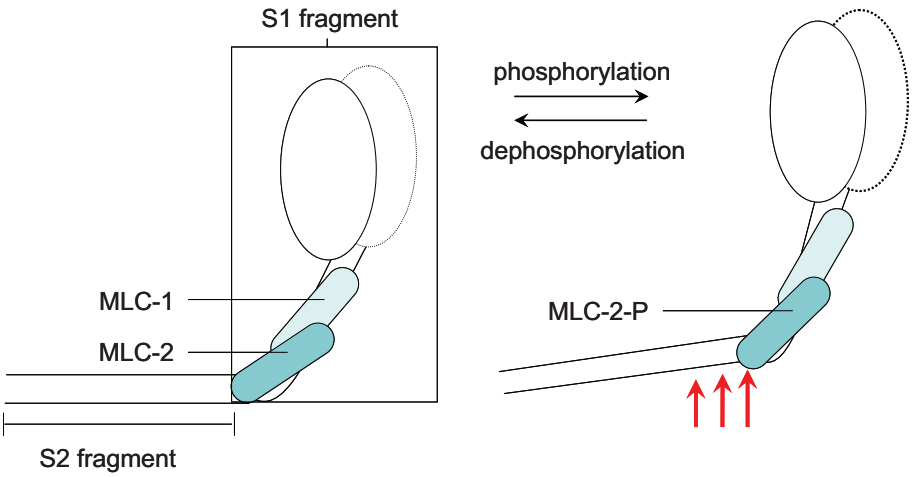

***Figure 2**: The mechanism of Ca^{2+} sensitization through MLC-2 phosphorylation. Red arrows indicate the elevation of the S1 fragment away from the myosin backbone and closer to the actin filaments.*

In cardiac tissue, MLC-2 phosphorylation (Ser^{15}) leads to modulation of cardiac muscle contraction. Positive inotropic effects after treatment with agonists for α_1-adrenoceptors (α_1-AR) [34], endothelin [35] and prostanoid F receptors [36], and for 5-

HT_{2A} [37] and muscarinic M_2 receptors [38] in normal and failing hearts, respectively, are thought to be dependent on MLC-2 phosphorylation. Thus, MLC-2 phosphorylation facilitates contraction and mediates inotropic responses to various neurohumoral stimuli in the heart. For comparison, in smooth muscle, contractility is *induced* by the phosphorylation of MLC-2 (Ser^{19}) [39].

In vivo hearts have been shown to exhibit a spatial gradient of decreasing vMLC-2 phosphorylation across the chamber wall from the epicardium and apex towards the endocardium, which is thought to facilitate a torsional contraction with higher contractile force at the wall surface than close to the cavity [40].

MLC-2 has also been identified as an essential component of sarcomere assembly and contractility in the vertebrate heart [41, 42]. Transgenic mice expressing a non-phosphorylatable ventricular isoform display no Ca^{2+}-senitization, and long-term effects involve generally reduced cardiac function [43, 44]. The identification of MLC-2 mutations linked to familial hypertrophic cardiomyopathy [45, 46] underscores the importance of understanding its actions as a regulator of contraction and cellular organization, and also understanding how the protein itself is regulated.

Protein kinases

Protein kinases are key regulators of cell function, and constitute one of the largest and most functionally diverse gene families. By removing a phosphate group from ATP and covalently attaching it to an amino acid that has a free hydroxyl group, providing a phosphate ester, they direct the activity, localization and overall function of many proteins, and serve to orchestrate the activity of almost all cellular processes [47]. Proteins can be phosphorylated on nine different amino acids (tyrosine, serine, threonine, cysteine, arginine, lysine, aspartate, glutamate and histidine) with serine, threonine and tyrosine phosphorylation being predominant in eukaryotic cells [48]. Protein kinases can be classified by the nature of their substrate. The by far largest group, serine/threonine specific kinases, can be further classified based on their activators; e.g. cAMP-dependent, cGMP-dependent, Ca^{2+}/calmodulin-dependent or Ca^{2+}/phospholipid-dependent (PKC). Each of these subclasses may include several isoenzymes [49].

Regulation and inhibition of protein kinases

Because protein kinases have profound effects on a cell, their activity is highly regulated. Kinases are turned on or off by 1) phosphorylation, 2) binding of activator or inhibitor proteins, 3) binding of small molecules, or 4) controlling their location in the cell relative to their substrates. Deregulated kinase activity is a frequent cause of disease, particularly cancer, since kinases regulate many aspects that control cell growth, movement and death [50].

Most kinase inhibitors discovered to date are ATP competitive and present one to three hydrogen bonds to the amino acids located in the hinge region of the target kinase, thereby mimicking the hydrogen bonds that are normally formed by the adenine ring of ATP. *Type 1 inhibitors,* constituting the majority of ATP-competitive inhibitors, recognize the so-called active conformation of the kinase, a conformation otherwise conducive to phosphotransfer. Unfortunately, the ATP-binding site within the serine/threonine kinase group displays striking homology. Inhibitors acting on this site are therefore unlikely to show a high degree of selectivity. *Type 2 inhibitors* recognize the inactive conformation of the kinase. *Allosteric inhibitors* bind outside the ATP-binding site, and modulate kinase activity in an allosteric manner. Inhibitors in this category tend to exhibit the highest degree of kinase selectivity because they exploit binding sites and regulatory mechanisms unique to a particular kinase. *Covalent inhibitors* are capable of forming an irreversible, covalent bond to the kinase active site, most frequently by reacting with a nucleophilic cysteine residue [47].

Myosin light chain kinase

Cardiac MLCK (cMLCK) was recently discovered as a new player in the regulation of MLC-2 in the heart [42, 51]. Before that, skeletal muscle MLCK (skMLCK) and smooth muscle MLCK (smMLCK) were thought to be responsible for the observed MLC-2 phosphorylation. A common feature of all three MLCKs is the dedication to one substrate: MLC-2 [16]. The smMLCK gene expresses three transcripts in a tissue-specific manner. The short form is expressed in smooth muscle, but also in cardiac muscle at lower levels. The long form is found in smooth muscle cells in culture, embryonic smooth muscle and non-muscle cells. The third transcript, containing only the C-terminal

immunoglobulin module, is expressed in phasic smooth muscle tissues [52]. Likewise, skMLCK is predominantly expressed in skeletal muscle, but also detected in cardiac muscle [40, 53], although its abundance has been controversial [54]. However, ablation of the skMLCK gene in mice resulted in no changes in cardiac MLC-2 phosphorylation, but the mice failed to increase MLC-2 phosphorylation in skeletal muscle in response to repetitive electric stimulation [55]. Similarly, ablation of the long form of smMLCK did not affect cardiac MLC-2 phosphorylation either [56]. Together, these findings lay the groundwork for an expectation of a cardiac isoform of MLCK.

Rat cMLCK (795 aa, 86 kDa) consists of a conserved kinase domain including an ATP-binding site at the C terminus, with 58% identity with skMLCK and 44% identity with smMLCK. However, the N terminal domain is unique, with no significant homology to other known proteins. It is expressed only in the heart; in both atrium and ventricle. In rat neonatal heart, it was expressed 9- to 18-fold more abundantly than smMLCK. It colocalized with actin, but surprisingly not with MLC-2. The most unexpected finding though, was the phosphorylation of MLC-2 in a Ca^{2+}/calmodulin-independent way [51], in contrast to the human cMLCK which was found to be Ca^{2+}/calmodulin-dependent [42]. Rat cMLCK had a high affinity and relatively low catalytic efficiency to MLC-2, suggesting it may serve to maintain basal MLCK activity in the heart [57]. This was supported by the fact that overexpression of cMLCK led to enhanced MLC-2 phosphorylation, whereas downregulation decreased steady-state level of MLC-2 phosphorylation. The striking difference in Ca^{2+}/calmodulin-dependency may simply be attributable to species differences, but in vitro assessment may not be sufficient to resolve this issue. Thus, we must await studies from genetically manipulated animals such as cMLCK deficient or overexpressing mice [57].

Protein phosphatases

Eukaryotic protein phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. The phosphoprotein phosphatase (PPP and PPM) families encode protein Ser/Thr phosphatases, whereas the protein tyrosine phosphatase (PTP) family includes both tyrosine-specific and dual-specificity (Ser/Thr and tyrosine) phosphatases. Within each superfamily, considerable structural diversity is

achieved by the attachment of regulatory and targeting domains and/or subunits to the protein catalytic domain, forming a variety of holoenzymes. PP1, PP2A and PP2B of the PPP family, together with PP2C of the PPM family, account for the majority of the Ser/Thr phosphatase activity in vivo [58].

Phosphatases are highly regulated proteins, but it is important to make a clear distinction between the terms “regulators” and “inhibitors”. Regulatory subunits serve to localize the enzyme to particular subcellular localizations and modulate protein selectivity. More specifically, the catalytic subunits can bind a host of regulators, and several heterotrimeric PP1 complexes have been observed [59, 60]. Organisms also express different regulatory subunits in different tissues at different times [61, 62], in addition to undergoing multi site phosphorylation with each site having a unique effect on substrate activity [63]. Inhibitors, on the other side, appear to inhibit phosphatase activity against a wider array of substrates [64], by mechanisms such as e.g. physical occlusion of the catalytic site [65].

PP1 and PP2A are strongly inhibited by a diverse array of natural occurring toxins, e.g. polyether polyketides such as okadaic acid [66, 67] and tautomycin [68], nonribosomal peptides represented by microcystin LR [69, 70], and polyketide/nonribosomal peptides such as calyculin A [71]. Calyculin A, initially isolated from the marine sponge Discodermia calyx, was found to inhibit PP1 and PP2A with IC_{50} values of 2 nM and 0.5-1.0 nM, respectively [72]. It consists of a polyketide and a dipeptide with a phosphate group in the polyketide portion (figure 3) [73]. X-ray crystallography has revealed that calyculin A binds to PP1 in a hydrophobic groove and an acidic groove on the surface of PP1. Calyculin A therefore recognizes PP1 by both hydrophobic and hydrophilic interactions, and the recognition site formed by the grooves consists of Arg96 and Arg221 [74].

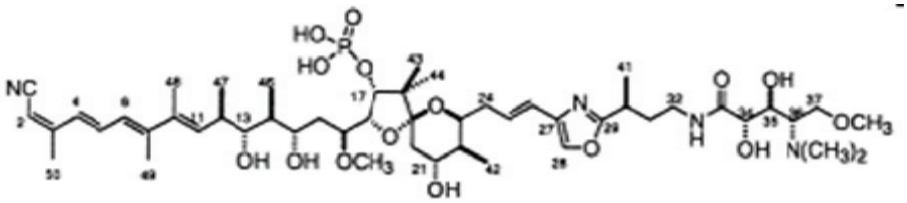

Figure 3*: Structural formula of calyculin A, a phosphatase 1 and 2A inhibitor. Adapted from Kita et al. 2002 [74].*

Myosin light chain phosphatase

MLCP is composed of three subunits: the catalytic subunit PP1c, a targeting subunit termed myosin phosphatase target subunit (MYPT) and a smaller subunit of unknown function. Most of the properties of myosin light chain phosphatase are due to MYPT and include binding of PP1c and substrate. The MYPT family consist of five molecules; MYPT1, MYPT2, MBS85, MYPT3 and TIMAP (TGF-β-inhibited membrane associated protein). MYPT1 is expressed in many tissues although present in higher concentrations in smooth muscle [75]. Some years ago, MYPT 2 (110 kDa) was cloned, and found to be present in heart and brain [76]. In mouse heart, the ratios of MYPT2:MYPT1:MBS85 were 10:2.7:1, respectively. Further characterization described the holoenzyme of cardiac/skeletal muscle MLCP (cMLCP) as composed of the catalytic subunit PP1cδ, and the two regulatory subunits HS-M_{21} and MYPT2 [77]. cMLCP was shown to display an overall identity of 61% with smMLCP, it was located close to the Z-line, it bound to the active form of RhoA, and was phosphorylated (Thr^{646}) by Rho kinase with associated inhibition of the MYPT2-PP1cδ complex.

The link between extracellular stimuli and cellular responses

Intracellular signalling coordinates life sustaining cellular processes as diverse as growth, differentiation, maintenance of specific cell functions, energy production, protective responses to various types of extracellular stressors, and, if rescue operations fail, even energy consuming programmed death by apoptosis. Stimuli for these various processes

reach the cell in the form of extracellular primary messengers like transmitters (e.g. acetycholine, norepinephrine, serotonin) and hormones (e.g. epinephrine, dopamine, angiotensin-II, endothelin, growth factors, cell mediators) via the blood [1]. Cellular responses like contractility of the heart and regulation of kinases and phosphatases are the consequences of transmission of the extracellular stimuli through G-protein-coupled receptors and G proteins.

G-protein-coupled receptors

G-protein-coupled receptors (GPCR, heptahelical receptors) are the single most diverse class of cell surface receptors, which bind the extracellular primary messengers. Characteristic for the GPCR superfamily of cell surface receptors are their seven transmembrane(TM)-spanning α-helices connected by three intracellular (i1-3) and three extracellular (e1-3) loops. Most GPCRs have two cysteine residues which form a disulfide bond important in packing and stabilising of a restricted number of conformations of these seven TMs. Aside from sequence variation, GPCRs differ in the length and function of their extracellular N-terminus, intracellular C-terminal tail, and their intracellular loops. Each of these domains provides specific properties. Besides transmission of extracellular signals, the GPCR's activity is modulated by cellular signals in an auto- or transregulatory fashion [78]. The transition from inactive to active conformation of the receptor is associated with a change in the relative orientation of the seven TMs, which reveals sites for G protein recognition and activation. The fine-tuning of their coupling to G proteins is regulated by splicing, RNA editing and phosphorylation. Some GPCRs have been found to form either homodimers or heterodimers with a structurally different GPCR, but also with membrane-bound proteins having one transmembrane domain. Finally, a few GPCRs are unfaithful to G proteins and interact directly, via their C-terminal domain, with proteins containing PDZ and Enabled/VASP homology (EVH)-like domains [78].

G proteins

Heterotrimeric guanine nucleotide-binding proteins (G proteins; subunits α, β and γ) couple the activation of GPCR receptors to the intracellular signalling pathways. As the primary intermediary between receptors and effectors, G proteins play a critical role in

determining the specificity and temporal characteristics of the cellular responses to a diverse array of extracellular stimuli [79]. Receptors catalyze exchange of tightly bound GDP for GTP on the α subunit in a process that requires the complete heterotrimer. This is the rate-limiting step in G protein activation. Once GDP is released, a stable, high-affinity complex between the activated receptor and G protein is formed. Binding of GTP to Gα destabilizes this complex allowing both subunits (Gα-GTP) and Gβγ to interact with downstream effector proteins. The activation is terminated by the hydrolysis of GTP by the GTPase activity inherent to the Gα subunit. The GDP-bound α subunit then reassociates with the βγ complex and is ready to enter a new activation cycle [80].

The G_α subunit classify G proteins into 4 primary families: G_s, $G_{q/11}$, $G_{12/13}$ and $G_{i/o}$. Common to G_s family members are their capability to activate adenylyl cyclases, and thereby causing an increase in 3',5'-cyclic adenosine monophosphate (cAMP). The G_s is ubiquitously expressed, and is known to couple to β_1- and β_2-adrenoceptors in the heart. $G_{q/11}$ is also ubiquitously expressed in mammals. A wide variety of receptors in the heart couple to this G protein family, including α_1-adrenoceptors, endothelin-, urotensin-II-, 5-HT_{2A}-, and angiotensin receptors, and they all result in an increased intracellular concentration of PLC-β. The $G_{12/13}$ family of heterotrimeric G proteins couple receptors to Rho guanine-nucleotide-exchange factors (RhoGEF) resulting in the activation of Rho A. Many GPCRs have been shown to mediate $G_{12/13}$ activation. For some receptors a selective coupling has been demonstrated, but with a few exceptions $G_{12/13}$-coupled receptors also interact with $G_{q/11}$, and, in some cases, also with G_i-type G proteins. Thus a typical feature of $G_{12/13}$-mediated signalling events is that they are usually initiated in parallel with $G_{q/11}$-mediated processes. $G_{i/o}$ are ubiquitously expressed and are classically associated with the inhibition of adenylyl cyclase (AC). Of the three G_i isoforms (G_{i1}, G_{i2}, G_{i3}), G_{i2} is the predominant isoform in cardiomyocytes. G_{i2} and G_{i3} have both been shown to couple to cardiac β_2-ARs [81]. G_i has also been suggested to couple to muscarinic M_2 receptors [38, 82].

AIMS OF THE THESIS

The overall aim of the thesis was to elucidate aspects of how phosphorylation of cardiac MLC-2 is regulated and its modulatory role in heart contraction. More specifically, we wanted to:

1. develop a method to estimate and evaluate the efficacy of selective enzyme inhibitors used in the study of cell signalling in cardiomyocytes/cardiac tissue (paper I)
2. study and compare which kinase activities are involved in the basal phosphorylation of MLC-2 in quiescent and electrically stimulated cardiomyocytes (paper II + III)
3. investigate the relevance of cultured primary cardiomyocytes as a model system for the intact beating heart muscle (paper II +III)
4. elucidate some aspects of the signalling pathways involved in agonist-induced GPCR-stimulation in cardiomyocytes, preferentially through $G_{q/11}$-coupled receptors (paper III)
5. investigate the role of MLC-2 phosphorylation in the positive inotropic effect observed after calyculin A stimulation (paper III) and muscarinic receptor activation (paper IV)

SUMMARY OF THE PAPERS INCLUDED

Paper I

In this paper, we presented and discussed a method for calculating the reaction rate constants and, thus, for evaluating the efficacy of one or more inhibitors when introduced to a forward-backward pair of enzymatic reactions. For example, in an apparent equilibrium process, reactant A can react into reactant B and vice versa, where E_1 is the enzyme converting A into B, and E_2 is the enzyme converting B into A. The dynamic steady state of the process is then dependent on the activities of E_1 and E_2. If one of the enzymes is selectively inhibited, the balance between the two enzyme activities is shifted and new apparent steady states are elicited thus making it possible to calculate the reaction rate constants.

As an example, reaction rate constants were calculated for the phosphorylation and dephosphorylation reaction of MLC-2 in isolated quiescent cardiomyocytes. The phosphorylation reaction rate was found to be one-fifth the rate constant of the dephosphorylation in basal steady state. However, in the presence of calyculin A, a phosphatase 1 and 2A inhibitor, this balance was shifted. The new apparent rate constant of the residual phosphatase activity was only one-third the rate constant of the kinase activity. Calyculin A (10^{-7} M) was also found to inhibit phosphatase activity by 93%, important knowledge in the further usage of this compound.

The method depends on knowing (i) the ratio $[A]_0/[B]_0$ in basal condition, and (ii) the changes occurring after the addition of the inhibitor. A prerequisite is that A and B are not subjected to any other conversions. The calculations are adjusted to apply for both zero-order and first-order enzyme kinetics. The method's foremost advantage is to provide valuable information when assessing reactions, and improve calculation and interpretation of generated data in a precise, easy and low-cost manner.

Paper II

The primary objective of paper II was to investigate which kinase activities are involved in the basal phosphorylation of MLC-2 in rat quiescent cardiomyocytes without agonists present. By maximally inhibiting MLCP, all changes in phosphorylation reflect the action of kinase activities. This, in combination with preincubating the cardiomyocytes with a non-selective kinase inhibitor, revealed a complex concentration-response curve. Having two components with IC_{50}-values separated by more than two log units, the curve clearly indicated two kinetically different groups of kinase activities. Preincubation in the absence and presence of various selective protein kinase inhibitors alone and in combinations indicated the presence of at least three active kinases; CaMKII, a Ca^{2+}/calmodulin-dependent and a Ca^{2+}/calmodulin-independent kinase activity. This is, to our knowledge, the first report that CaMKII phosphorylates MLC-2 in cardiomyocytes. The surprising lack of effect of two different MLCK inhibitors suggested an insignificant role of MLCK, the enzyme so far believed to be the only MLC-2 kinase operating in the heart. We also conclude that neither PKC nor Rho kinase phosphorylate MLC-2 in quiescent cardiomyocytes.

Paper III

In paper III we investigated aspects of *in situ* MLC-2 phosphorylation in models of electrically stimulated adult rat cardiomyocytes in culture and rat ventricular muscle strips *ex vivo*. Previous findings (paper II) in quiescent cardiomyocytes demonstrated a lack of MLCK activity, but a significant role for CaMKII in combination with two unidentified kinase activities not caused by PKC or Rho kinase. Here we show how MLCK is recruited in electrically stimulated cardiomyocytes in the presence of Ca^{2+} transients, by observing a 41.7% reduction in MLC-2 phosphorylation reaction rate after selective MLCK inhibition (by ML-7). CaMKII, identified by selective inhibition by KN-93, was also found to act as an MLC-2 kinase in this model, together with an unidentified Ca^{2+}/calmodulin-independent kinase activity insensitive to staurosporine and PKC inhibition. The kinase activities were revealed under maximal phosphatase inhibition by calyculin A (10^{-7} M).

No significant effects of agonists (phenylephrine, fluprostenol and carbachol) were observed in the cardiomyocytes beyond the effect of calyculin A, although the apparent numerical ML-7 sensitivity increased in the presence of phenylephrine and fluprostenol. By comparison, calyculin A induced a sustained positive inotropic effect in muscle strips which could be abolished by ML-7 preincubation. The inotropic effect correlated with levels of MLC-2 phosphorylation as measured in frozen preparations. Taken together, the present results clearly demonstrate a key role for MLCK as an MLC-2 kinase in both electrically stimulated cardiomyocytes and muscle strips. The latter model also suggests a role for MLC-2 phosphorylation in mediating calyculin A-induced increase in contraction.

Paper IV

The study in paper IV was designed to characterize an observed carbachol-induced positive inotropic effect in ventricular muscle from rats with congestive heart failure, and elucidate the mechanism of action. The inotropic response, which was not seen in sham-operated rats, did not correlate with infarction size, but did correlate with increased left ventricular end diastolic pressure, heart weight/body weight ratio and lung weight, primary indicators of the severity of heart failure. Antagonizing the carbachol-evoked response produced the expected potency shift only when using selective M_2-receptor antagonists. The response was also abolished by pertussis toxin pretreatment, an indicator of G_i-coupling and hence further supporting a role for the M_2 receptor in the mediation of the inotropic response. The inotropic effect revealed an increase in myofilament Ca^{2+}-sensitivity through increased MLC-2 phosphorylation, which could be reversed by inhibitors of both Rho kinase and MLCK.

This is the first report to show that activation of muscarinic receptors, most likely M_2 receptors, increased contractility in failing but not sham-operated rat ventricles, by increasing MLC-2 phosphorylation. Shifting to this less energy-demanding mechanism of inotropic support may be particularly advantageous in failing hearts.

RESULTS AND DISCUSSION

Methodological considerations

Cell cultures

All cells and muscle tissue used in this thesis originate from male Wistar rats (out-bread strain). The final cell cultures consisted of >95% ventricular, elongated, striated, non-contracting cardiomyocytes as observed in the microscope (figure 4). Due to gradient centrifugation during the isolation protocol, contamination from fibroblasts was not a problem, and the last <5% were mainly rounded-up cardiomyocytes.

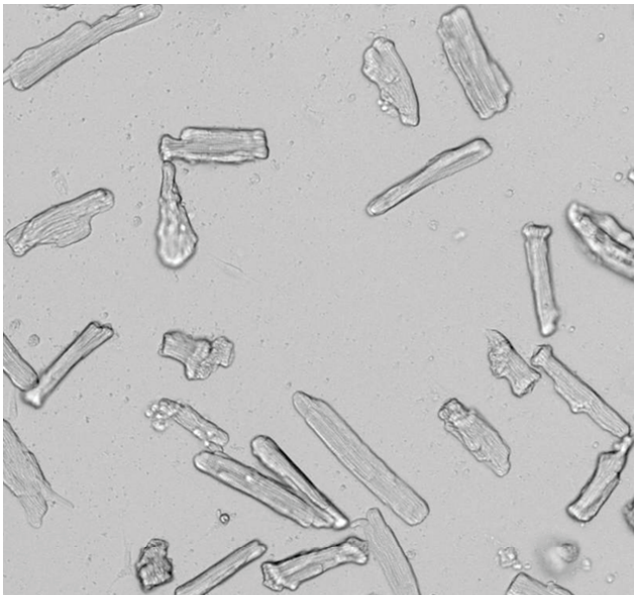

Figure 4*: Adult rat ventricular cardiomyocytes in culture. Photographed by H. Eikemo.*

There are several advantages in using cell cultures when studying regulation of cardiac function and signalling. Firstly, it is a widely used model system which makes it comparable between different laboratories. Secondly, one does not have to deal with neurotransmitter release observed in whole muscle tissue sample, which facilitates studies on basal steady-state conditions. Also, a high number of interventions can potentially be executed on the same cell population, controlling nicely for variation within the assay and facilitating paired comparisons. Disadvantages include the un-physiological state by not having i) cell-cell-junctions, ii) an intact extra-cellular matrix (peeled off during collagenase treatment), and iii) contact with e.g. fibroblasts and other cell types normally interacting together. Studies on co-cultures between cardiomyocytes and fibroblasts have been performed and compared to monocultures. The results showed a different response-pattern, indicating that the cross-talk between cell types is not unimportant [83].

In isolated quiescent adult cardiomyocytes, as used in papers I and II, the concentration of intracellular Ca^{2+} is fairly stable without Ca^{2+}-transients, and no spontaneous contraction is observed. This situation of cellular components makes it a good model for disclosing and studying Ca^{2+}-transient-independent processes *in situ* which may otherwise be masked by or secondary to Ca^{2+} transient-dependent processes in the contracting cardiomyocyte or beating myocardium. Experiments on quiescent cells were performed ~20 h after isolation, whereas studies on contracting cells (paper III) were performed on newly isolated cells (<3 h). Apparently, the cardiomyocytes lost the ability to respond to electrical stimulation after a period of 20 h quiescence.

Observing electrically stimulated cardiomyocytes in the microscope during kinase inhibition, striking dissimilarities were seen with regard to morphology and contraction pattern. After 40 min of calyculin A incubation, ~50% of control cells (no kinase inhibitor present) had disturbed morphology (cells were rounded up) and irregular contraction pattern. W7 preincubation preserved an intact morphology, but removed all contraction. ML-7 preincubation however, caused a marked loss of the cell's striated and elongated form, and also influenced the contraction by partly inhibiting it and partly making it irregular. So, it seems that calmodulin inhibition protects the cells from losing their original morphology, whereas MLCK inhibition speeds up the process of sarcomere

disorganization/disruption. This is in line with previous reports demonstrating an essential role of MLCK in sarcomere assembly [41, 42].

Another interesting observation was how the cells preserved their morphology and contractility when electrical stimulation was started simultaneously with calyculin A addition. If electrical stimulation was started 5 min prior to calyculin A addition, which was the case in all experiments in paper III, the cells had a much higher tendency of rounding up and displaying a fibrillation-like contraction. These observations indicate a protective role of phosphatase inhibition. Nevertheless, the latter protocol was chosen since the cardiomyocytes needed approximately 4-5 min to get into rhythmical contraction after the onset of electrical stimulation. Furthermore, the cells lost their morphology if electrical stimulation was applied during all of the intervention time (i.e. during 45 min of kinase inhibition followed by up to 40 min of phosphatase inhibition). Therefore, starting electrical stimulation 5 min prior to calyculin A addition was a compromise.

Muscle strips

In papers III and IV, isometrically contracting left ventricular muscle strips were used [84]. Most cell types present in the ventricle is also present in this multicellular preparation. Thus the structural composition of the heart muscle tissue is intact with preserved cell-to-cell connections and an extracellular matrix. Despite the presence of non-cardiomyocytes, the functional mechanical response measured is cardiomyocyte-specific. Taken together, muscle strips might represent a more physiologically relevant model system mimicking the intact beating heart, although extrapolation to *in vivo* conditions must be done with great caution.

Western blotting and quantification

Although not expressing absolute amounts of MLC-2 phosphorylation, the MLC-2 phosphorylation assay has several advantages. The antibody used is highly vMLC-2 specific and identifies both phosphorylated and non-phosphorylated forms simultaneously (recognizes residue 45 to 59, far away from the phosphorylated residue 15) without the need to strip the membranes and hence risk losing some of the signal (figure 5). Furthermore, by calculating MLC-2 phosphorylated in relation to total MLC-2

within each sample, any potential differences in amount of protein loaded from lane to lane will be eradicated, thus giving a more accurate estimate. This was confirmed by assaying dilution series of the samples which produced similar results (data not shown).

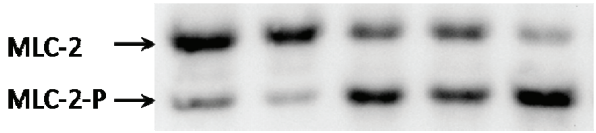

Figure 5*: Representative western blot where both phosphorylated and non-phosphorylated MLC-2 have been identified.*

Percentile plots

The percentile plot is obtained by normalizing the effector variable (e.g. MLC-2 phosphorylation) from each experiment between 0 and 100, and the resulting values are used to calculate the percentile effect at each actual point of measurement (=x-axis, e.g. time or drug concentration). The curve is based on the x,y pairs corresponding to defined percentiles of the effector variable (e.g. 10, 20, 30 etc. percent) and plotted with the resulting curve, for illustration. This means that e.g. the x value of the 50 percentile indicates the time to half maximal effect [85]. The foremost advantage of using percentile plots is how it normalizes a set of experiments and makes them directly comparable even though e.g. basal values might vary. The method provides the curves with the appropriate slope which otherwise might be artificially shallow when estimated by simply averaging the responses at each concentration.

Specificity and selectivity of protein kinase and phosphatase inhibitors

There is considerable interest in the exploitation of selective protein kinase inhibitors for cell signalling studies. Many compounds reported to be selective have been examined and found to be so non-specific as to render meaningless any conclusions drawn from their use [86, 87]. In paper I we presented a method for estimating and evaluating the

efficacy of selective enzyme inhibitors, and demonstrated it on data from calyculin A-induced MLC-2 phosphorylation. We found that in our model system of quiescent cells, 10^{-7} M calyculin A inhibits MLCP 93%, which is indeed satisfactory from an experimental point of view. Staurosporine on the other hand, reduced MLC-2 phosphorylation from 16.1%±1.8% (absence of staurosporine) to 9.7%±1.0% (presence of staurosporine), inhibiting only 47% before adding the phosphatase inhibitor. However, one must be careful when applying the method to such low numbers where minor changes have a big impact on the outcome. Interpretation of the results should always be seen in the light of the biology of the system.

Table 1 gives an overview of reported selectivity of the kinase inhibitors used in this thesis. All of the inhibitors are cell membrane permeable. Calyculin A binds reversibly to the catalytic site of MLCP, which is classified as a protein phosphatase 1 [72]. BIM is a selective inhibitor of all PKC isoforms, with a ranked order of potency (α>β>ε>δ>ζ) [88].

Table 1*: Inhibitory constants for the various protein kinase inhibitors. All values are given in μM as K_i or, if indicated with *, as IC_{50}.*

	MLCK	**PKA**	**PKC**	**PKG**	**CaMKII**	**Rho kinase**
ML-9	3.8[89, 90]	32[90]	54[90]			#
ML-7	0.3[89]					
Stauro-sporine	*0.01[49] *0.021[91]	*0.015 [92]	*0.0027[93] *0.006[92]	*0.018[94]	*0.02[95]	
Y27632	>250[96]	25[96]	26[96]			0.14[96] *0.8[87]
W7	*25[97] 110[89]					
BIM			0.005-0.07[88]			
KN-93					*1[98]	

#: 100 μM of ML-9 reduces the kinase activity of Rho kinase by 77%, and 75 % and 46 % for smMLCK and skMLCK, respectively [86].

Kinases involved in the phosphorylation of MLC-2

By fully inhibiting MLCP by calyculin A (93% effective), as demonstrated in paper I, kinase activities involved in the phosphorylation of MLC-2 were successfully revealed. Maximal calyculin A-induced MLC-2 phosphorylation was roughly 60% and 80% in quiescent and contracting cardiomyocytes, respectively, and the reaction rate between 0 and 40 min of calyculin A incubation was doubled (figure 6). This clearly demonstrates a higher general kinase activity in working cells, as would be expected in muscle strips *ex vivo* and in the intact beating heart *in vivo*.

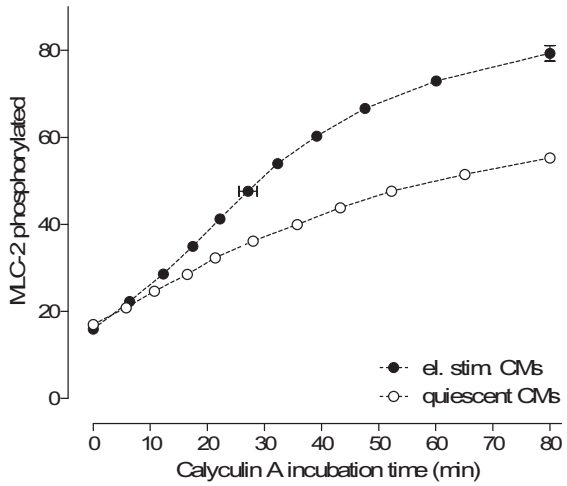

Figure 6*: The effect of maximal phosphatase inhibition by calyculin A in electrically stimulated vs. quiescent cardiomyocytes (CMs). Adapted from paper III.*

One of the main findings in this thesis is that MLCK is recruited *in situ* going from a model of quiescent to contracting cells. From being completely inactive in resting cells, as demonstrated by a lack of effect of both ML-7 and ML-9, inhibition of MLCK accounted for a reduction of 41.7%±0.1% in MLC-2 phosphorylation reaction rate in electrically stimulated cells. Quiescent cells do not experience Ca^{2+}-transients, but instead

have a relatively stable $[Ca^{2+}]_i$ of around 100 nM. Absence or presence of Ca^{2+}-transients is perhaps the key difference between the two model systems, and therefore likely the main reason for the recruitment of MLCK. However, the substantial effect of CaMKII inhibition also in quiescent cells suggested a role for Ca^{2+}/calmodulin-dependent activity despite the absence of Ca^{2+}-transients. These observations indicated that MLCK and CaMKII might have different requirements as to Ca^{2+}-activating profiles. MLCK requires Ca^{2+} transients, whereas for CaMKII it is sufficient with a steady-state resting level of Ca^{2+}. To our knowledge, papers II and III are the first reports demonstrating a role for CaMKII-mediated phosphorylation of MLC-2 in cardiomyocytes.

Findings from papers II and III clearly demonstrate that different kinases are actively phosphorylating MLC-2 in different model systems. Two kinases remain un-identified in quiescent cells (paper II): one that is Ca^{2+}/calmodulin-dependent, and one that is not. Rho kinase, PKC and MLCK were ruled out as active kinases phosphorylating MLC-2. In electrically stimulated cells (paper III), a Ca^{2+}/calmodulin-independent kinase activity remained unidentified. Therefore, looking to smooth muscle MLC-2 kinases, which have been much more extensively investigated than cardiac, might be helpful in getting some clues. Table 2 summarizes what is known so far in smooth muscle.

The effect of CaMKII was described in paper II as a direct phosphorylation of MLC-2. However, CaMKII might also phosphorylate MLCK [99, 100]. In smMLCK, phosphorylation by CaMKII at a specific serine residue (site A) near the calmodulin-binding domain, reduces the affinity of MLCK for Ca^{2+}/calmodulin, indirectly reducing the phosphorylation of MLC-2 by MLCK [101]. The net effect of these two mechanisms is complex to predict, as it proposes that a Ca^{2+}-dependent MLCK phosphorylation inhibits a Ca^{2+}-dependent MLCK activity. However, it seems like the Ca^{2+} concentration partly decides which mechanism dominates, with higher concentrations needed for MLCK phosphorylation [102].

***Table 2**: MLC-2 kinases in smooth muscle.*

KINASE	PHOSPHORYLATION SITE	Ca^{2+} DEPENDENCY	REFERENCE
MLCK	$Ser^{19}>Thr^{18}$	+	[103]
Integrin-linked kinase (ILK)	$Ser^{19}=Thr^{18}$	-	[104]
Zipper-interacting protein kinase (ZIPK)	$Ser^{19}=Thr^{18}$	-	[105]
Rho kinase	Ser^{19}	-	[106]
p21-activated kinase β (PAK3)	Ser^{19}	-	[107]
CaMKII	Ser^{19}	+	[108]
p21-activated kinase γ (PAK2)	Ser^{19}	-	[109]
MAPKAP2	Ser^{19}	-	[110]
p90 ribosomal s6 kinase(RSK)-2	Ser^{19}	-	[111]
AIM-1	Ser^{19}	-	[112]

Applying the theory described above to our data on quiescent cells, it can be hypothesized that low Ca^{2+} concentrations facilitate CaMKII-mediated MLC-2 phosphorylation, and have no effect on MLCK phosphorylation nor MLCK recruitment. KN-93 would then reduce MLC-2 phosphorylation as opposed to ML-7. Indeed, this is in agreement with our observations. If we assume that increasing the Ca^{2+} concentrations, as is the case in electrically stimulated cells, enables CaMKII-mediated phosphorylation of both MLC-2 and MLCK but with a net effect of the former, then KN-93 would still reduce MLC-2 phosphorylation. At the same time, there would be a significant Ca^{2+} activation of MLCK laying the grounds for an effect of ML-7. This is in accordance with what we observed in the model of contracting cardiomyocytes (paper III). A further

reduction in MLC-2 phosphorylation was achieved by combining KN-93 and ML-7, although not an additive effect, and not larger than the reduction caused by staurosporine. The latter fits nicely with the fact that staurosporine is supposed to inhibit both CaMKII and MLCK at the concentrations used in our experiments.

The involvement of RSK-2 (table 2) can probably be excluded in this context since BIM is known to inhibit this kinase with IC_{50} values in the nanomolar range [113, 114], and our data suggested no effects of BIM in neither quiescent nor contracting cells. As mentioned in paper II, staurosporine inhibits the Ca^{2+}-independent ILK [115], ZIPK [105, 116] and PAK3 [117], all with IC_{50} values in the nanomolar range. In the quiescent cells, these kinases did probably not participate as MLC-2 kinases, as they would all fall into the fraction of high sensitivity to staurosporine which was found to be CaMKII-mediated. In the contracting cells, the staurosporine-sensitive kinase activity had the same magnitude as ML-7 and KN-93 inhibition combined, suggesting no role for other staurosporine-sensitive kinases.

Rho kinase as a direct MLC-2 kinase was ruled out due to lack of effect of the selective inhibitor Y27632 in quiescent cells, and was not investigated in contracting cells. Documented effects of Rho kinase in cardiac cells consist of phosphorylation of the MLCP subunit MYPT, thereby inhibiting MLCP and hence increasing MLC-2 phosphorylation [118]. However, since our kinase activity assay involved complete phosphatase inhibition, the latter effect of Rho kinase was not identified.

PAK2 has been shown to phosphorylate non-muscle MLC-2 *in vitro* [109], and MLCK in endothelial cells [119], and MAPKAP2 and AIM-1 have not been linked to cardiac tissue, thereby rendering their role in heart cells less likely than the previously described alternatives.

So which kinases are responsible for the observed MLC-2 kinase activities so far not identified? Far from all candidate MLC-2 kinases described in smooth muscle have been investigated in cardiac cells. One might speculate that some of the kinase activity might be due to the presence of cardiac specific kinases. Furthermore, the concept of indirect MLC-2 phosphorylation through regulation of direct MLC-2 kinases, as suggested for CaMKII, is also a possibility.

In the context of unidentified kinases, it is tempting to bring up the identification of rat cMLCK which was, highly unexpectedly, found to phosphorylate MLC-2 in a Ca^{2+}/calmodulin-independent way [51]. As mentioned in the introduction, cMLCK had a high affinity and relatively low catalytic efficiency towards MLC-2. This was suggested by the authors to serve to maintain basal MLCK activity in the heart, a suggestion supported by the fact that overexpression of cMLCK led to enhanced MLC-2 phosphorylation, whereas downregulation decreased steady-state level of MLC-2 phosphorylation. In the studies conducted in this thesis, we have assumed that ML-7 inhibits all MLCK activity, and that MLCK activity is Ca^{2+}/calmodulin-dependent. But if the cardiac isoform really is Ca^{2+}/calmodulin-independent, and could escape the inhibitory effect (of both ML-7, ML-9 and staurosporine) due to structural deviation from the other MLCKs, it renders it as a hot candidate for the Ca^{2+}/calmodulin-independent kinase activity.

The discussion of MLC-2 kinases involved in quiescent and contracting cardiomyocytes has been summarized in figures 7 and 8.

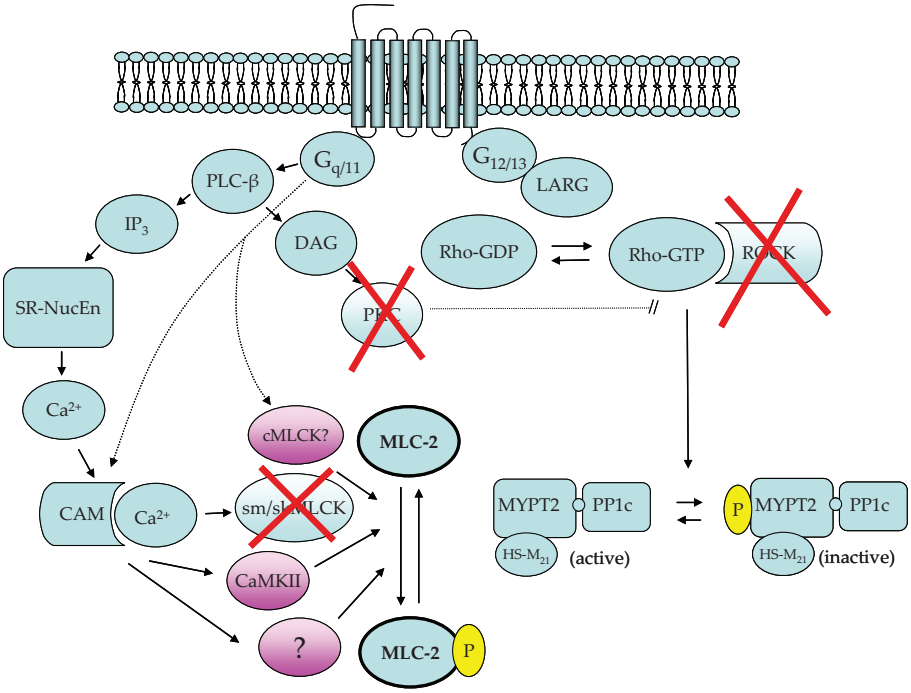

***Figure 7**: Known (solid lines) and hypothesized (dotted lines) signalling pathways in the regulation of MLC-2 phosphorylation in quiescent cardiomyocytes. Red crosses indicate kinases ruled out as direct MLC-2 kinases by lack of effect of selective inhibitors.*

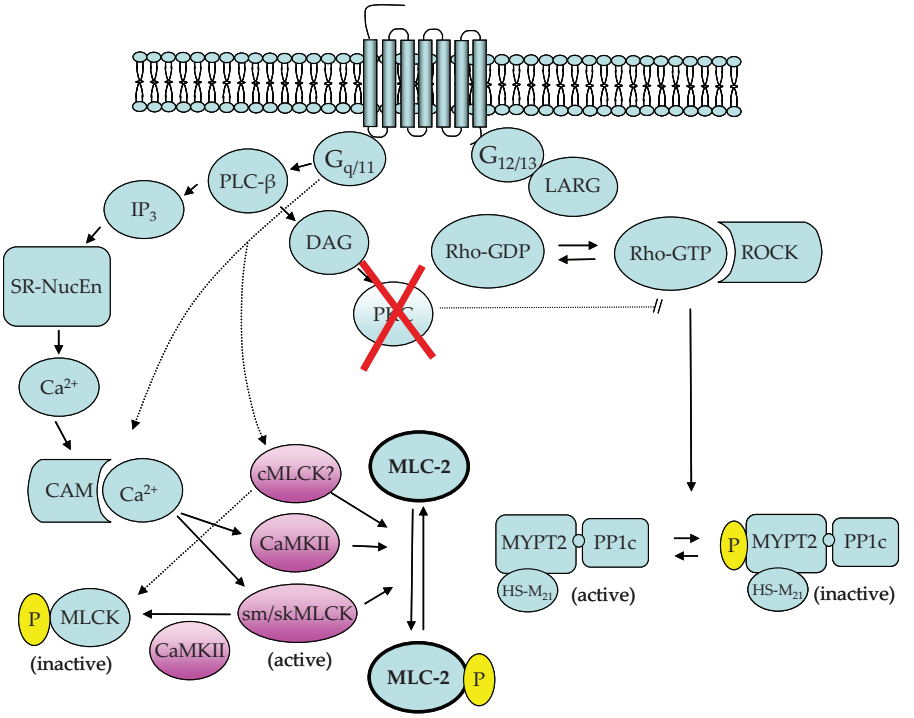

***Figure 8**: Known (solid lines) and hypothesized (dotted lines) signalling pathways in the regulation of MLC-2 phosphorylation in electrically stimulated contracting cardiomyocytes. The red cross indicates that PKC is ruled out as a direct MLC-2 kinase by lack of effect of BIM. Rho kinase was not studied in this model.*

Role of MLC-2 phosphorylation in modulation of contraction

Phosphorylation of Ser^{15} in MLC-2 [120] has been well documented to mediate Ca^{2+}-sensitization [33, 121, 122], and several studies suggest a role for this mechanism in modulation of contraction. Positive inotropic effects after treatment with agonists for α_1-adrenoceptors (α_1-AR) [34, 123], endothelin [35] and prostanoid F receptors [36] in normal hearts, and for 5-HT_{2A} [37] and muscarinic M_2 receptors [38] in failing hearts, are thought to be dependent on increased MLC-2 phosphorylation.

Calyculin A-induced positive inotropic effect in muscle strips

In ventricular muscle strips *ex vivo*, calyculin A alone elicited a sustained positive inotropic response which was blunted by pre-treating the strips with ML-7. This suggests that the calyculin A-induced inotropic response is caused by MLCK-mediated phosphorylation of MLC-2. ML-7 alone did not lower basal contractile force, in contrast to the combined effect of ML-7 and calyculin A. The contraction-relaxation curves during the positive inotropic response to calyculin A revealed no change in time to peak force nor the relaxation time compared to basal. These characteristics are typical of G_q-mediated agonist responses as previously shown for e.g. α_1-adrenergic receptors [84]. Especially the lack of a lusitropic effect is in sharp contrast to cAMP-mediated responses in the heart.

MLC-2 phosphorylation levels were also determined in the same muscle strips as inotropic responses were recorded, and the two variables were found to correlate. In parallel with the inotropic effect, calyculin A increased MLC-2 phosphorylation. ML-7 alone did not significantly reduce phosphorylation levels compared to time-matched control, in contrast to the combination of ML-7 and calyculin A. A correlation between inotropic effect and MLC-2 phosphorylation levels has to our knowledge not previously been shown in ventricle, and is consistent with a key role for MLC-2 phosphorylation in mediating positive inotropic effects in muscle strips.

The relevance of small MLC-2 phosphorylation differences

The level of basal MLC-2 phosphorylation has been reported to vary widely, ranging from 5% to 46% in mammalian ventricular myocardium [33, 121, 124]. Several studies on contracting muscle strips have demonstrated an *in situ* basal MLC-2 phosphorylation level of about 20% [36, 37], corresponding nicely with our results. The maximal *in vivo* MLC-2 phosphorylation has been reported to be around 30-40% [20, 125]. In absolute numbers, such an increase appears small. However, the calyculin A-induced MLC-2 phosphorylation in our cell culture model demonstrates a huge capacity of this signalling mechanism to respond to various neurohumoral stimuli. Reversible reactions in most biological systems display a maximum effect of 80%. Assuming the phosphorylation of MLC-2 follows the Michaelis-Menten equation, the steepest part of the phosphorylation

curve is between 20% and 80% phosphorylation. In this interval, small differences in kinase activity cause relatively large changes in phosphorylation, and are therefore most suited for regulation in a biological system. Likewise, if contractile force is plotted against MLC-2 phosphorylation levels, and the regulation is set on the steepest part of the curve, even minor changes in phosphorylation levels could give substantial effects.

Agonist effects in contracting cells and muscle strips

Paper IV demonstrated that activation of muscarinic M_2 receptors elicited positive inotropic effects in muscle strips from rat ventricle with heart failure and not in sham-operated animals. These effects were found to be mediated by MLC-2 phosphorylation by direct regulation of MLCK and indirect regulation of MLCP through Rho kinase. Inhibiting Rho kinase and MLCK separately attenuated the inotropic response by roughly the same magnitude (~70% reduction), and the increase in MLC-2 phosphorylation accompanying carbachol stimulation was abolished by both blockers separately.

Inotropic responses to muscarinic agonists in the heart have previously been reported in both normal human ventricular trabeculae [126], guinea pig papillary muscles [127, 128], embryonic chick ventricle [129, 130] and rat atria [128, 131], but this is the first report of such in the rat ventricle. Additionally, in normal rat heart, carbachol has been found to increase Ca^{2+} sensitivity with no apparent corresponding inotropic response [132, 133].

The muscarinic M_2 response was in paper IV characterized by a biphasic inotropic response composed of an initial relatively fast transient negative component followed by a slowly developing positive component. Also, it displayed a symmetrical change in the contraction-relaxation cycle with slightly prolonged time to peak tension and relaxation time. These are all typical features of effects mediated through G_q-coupled receptors, known to elicit Ca^{2+}-sensitization of the myofilaments [133] as seen with stimulation of α_1-adrenoceptors [34, 123, 134], 5-HT_{2A}- [37] and endothelin receptors [35], in addition to the calyculin A-induced MLC-2 phosphorylation observed in paper III. G_q is known to activate the Rho kinase pathway [135] exerting its effects through MLCP inhibition, thus increasing MLC-2 phosphorylation [96]. Y27632, a selective Rho kinase inhibitor, attenuated the carbachol-mediated positive inotropic effect and MLC-2 phosphorylation

when added before carbachol. In contrast, Y27632 did not inhibit the cAMP-dependent β-adrenoceptor-mediated inotropic response, consistent with previous findings [34, 37]. ML-9, which inhibits MLCK and Rho kinase to the same degree, had about the same effect as Y27632 upon the inotropic response and MLC-2 phosphorylation, indicating that these effects are mediated also through MLCK activity.

However, the fact that the carbachol-mediated positive inotropic response was *Pertussis* toxin-sensitive lends support to a signalling pathway going through G_i. Perhaps there is a switch in signalling pathways in the transition from normal to failing rat ventricle like reported in neonatal rat cardiomyocyte-derived H10 cells [136]. Using a less energy-demanding mechanism of inotropic support may be advantageous in the failing heart, as compared to cAMP-mediated mechanisms. An enhancement in Ca^{2+} sensitivity is therefore a reasonable method of doing so, but clearly further investigations are needed to clarify the functional role of carbachol-mediated inotropic response and which signalling mechanisms are involved.

Electrically stimulated cardiomyocytes (paper III) were treated with three different agonists. These were phenylephrine, fluprostenol and carbachol, acting on α_1-adrenergic, prostanoid F and muscarinic M_2 receptors, respectively. Agonist stimulation in the presence of calyculin A did not increase MLC-2 phosphorylation beyond the effect of calyculin A alone, although a tendency towards a slight elevation with phenylephrine and fluprostenol was observed. This led us to believe that agonist effects might be acting predominantly through modulation of MLCP activity, but agonist stimulation in the absence of calyculin A did not significantly increase MLC-2 phosphorylation, rendering the question rather open. The lack of effect of carbachol was, however, in line with what was observed in carbachol-stimulated muscle strips from sham operated animals (paper IV).

In the presence of calyculin A, ML-7 preincubation reduced MLC-2 phosphorylation levels in cardiomyocytes more when combined with agonists than without. This slight increase in ML-7 sensitivity is indicative of an increased MLCK activity in the presence of phenylephrine and fluprostenol. One might speculate if these agonists initiate signalling pathways increasing MLCK activity without being reflected in increased MLC-2 phosphorylation *per se*. Alternatively, lack of agonist effects could be

the result of a methodological problem if the collagenase treatment during the isolation procedure disturbed the receptors or in worst case peeled off the receptors. Further experiments are definitely needed to clarify agonist effects in electrically stimulated cardiomyocytes.

SUMMARY AND CONCLUSIONS

In the papers presented in this thesis we have investigated several aspects of MLC-2 phosphorylation, its regulation and significance in modulating cardiac contractility. Firstly, we presented a novel method for calculating reaction rate constants, demonstrated how to employ it and thus evaluate enzyme inhibitor efficacy. Secondly, quiescent cardiomyocytes were compared to electrically stimulated cells with respect to kinase activities involved in the phosphorylation of MLC-2. CaMKII was found to play a key role in this phosphorylation reaction when the cells are not contracting, but MLCK dominates when the cells contract. Furthermore, unidentified kinase activities were also detected, both Ca^{2+}/calmodulin-dependent and –independent present in quiescent cells, and Ca^{2+}/calmodulin-independent present in contracting cells. Agonist stimulation of contracting cells did not significantly elevate MLC-2 phosphorylation, but muscarinic M_2 receptor stimulation was found to be mediated through MLC-2 phosphorylation in muscle strips from failing rat hearts. Additionally, calyculin A-induced positive inotropic effects in normal rat ventricle strips were also found to be mediated through MLC-2 phosphorylation. Finally, after using three different model systems (quiescent cells, contracting cells and contracting muscle strips), we observed apparent differences between the different models with respect to agonist stimulation and regulation of the level of MLC-2 phosphorylation. This indicates that the regulation of the MLC-2 phosphorylation level is very dynamic and difficult to reveal. The results also indicate that several enzymes together serve to guarantee at least a certain minimum level of MLC-2 phosphorylation during various conditions. The study as a whole clearly demonstrates the weakness of using only one model system in approaching such questions.

FUTURE PERSPECTIVES

The current research has revealed new aspects of how contractile function is regulated, but it has also triggered several new questions which would be highly interesting to pursue. The most intriguing question is how different kinases seem to switch between different activity levels depending on the state of the cell. In future studies, it would be interesting to further investigate which kinases are involved in the various model systems used for the study of the intact beating heart *in vivo*. Clearly, there are essential differences depending on whether the model is e.g. cells or tissue, neonatal or adult, normal or failing. One way of approaching this question would be to knock out all MLCK isoforms in the heart, and see whether other kinases would take over in electrically stimulated cells thus behaving like quiescent cells with respect to MLC-2 phosphorylation activity. On the other hand, as such an intervention could be detrimental at an early stage in the development of the animal due to no sarcomere assembly and formation and therefore disintegrated cells, this might necessitate the use of inducible knock-out.

Now when a cardiac MLCK has been described, several issues have to be resolved. Considering whether cMLCK is Ca^{2+}-dependent or not, is one basic question. An overwhelming amount of literature states that rat smMLCK and skMLCK are Ca^{2+}-regulated, and one study states that rat cMLCK is not [51]. The selectivity of inhibitors on cardiac isoforms needs to be clarified. If necessary, cardiac specific inhibitors should be designed.

The last three papers in this thesis described MLC-2 kinase activity in various model systems. However, the regulation of MLC-2 phosphorylation by phosphatase activity is also highly interesting, but was beyond the scope of this work. MYPT2, the regulatory subunit of cMLCP, has several phosphorylation sites displaying both stimulatory and inhibitory actions upon MLC-2 phosphorylation. As indicated in paper I,

the dephosphorylation reaction is by far the dominating in basal steady-state equilibrium therefore representing an enormous regulatory potential.

Further studies are also needed to resolve whether there are multiple functionally important phosphorylation sites on MLC-2. The protein contains several serine and threonine residues representing potential sites for phosphorylation. Mice expressing non-phosphorylatable MLC-2 have been made [43], and it will be exciting to see the results of e.g. functional contraction studies, agonist effects, and possible compensatory mechanisms on these animals. It is also important to look towards other sarcomeric proteins, with respect to their role and cooperativity in regulating cardiac contraction. Some of these proteins have only recently received attention, and much work remains.

REFERENCES

[1] Schaub MC, Hefti MA, Zaugg M. Integration of calcium with the signaling network in cardiac myocytes. J Mol Cell Cardiol 2006;41:183-214.

[2] Katz AM. Physiology of the heart. Lippincott Williams and Wilkins; 2006.

[3] Berg JS, Powell BC, Cheney RE. A millennial myosin census. Molecular Biology of the Cell 2001;12:780-94.

[4] Bahler M. Are class III and class IX myosins motorized signalling molecules? Biochim Biophys Acta 2000;1496:52-9.

[5] Yin H, Pruyne D, Huffaker TC, Bretscher A. Myosin V orientates the mitotic spindle in yeast. Nature 2000;406:1013-5.

[6] McNally EM, Kraft R, Bravo-Zehnder M, Taylor DA, Leinwand LA. Full-length rat alpha and beta cardiac myosin heavy chain sequences. Comparisons suggest a molecular basis for functional differences. J Mol Biol 1989;210:665-71.

[7] Hamilton N, Ianuzzo CD. Contractile and calcium regulating capacities of myocardia of different sized mammals scale with resting heart rate. Mol Cell Biochem 1991;106:133-41.

[8] Litten RZ, III, Martin BJ, Low RB, Alpert NR. Altered myosin isozyme patterns from pressure-overloaded and thyrotoxic hypertrophied rabbit hearts. Circ Res 1982;50:856-64.

[9] Lompre AM, Nadal-Ginard B, Mahdavi V. Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated. J Biol Chem 1984;259:6437-46.

[10] Mercadier JJ, Bouveret P, Gorza L, Schiaffino S, Clark WA, Zak R et al. Myosin isoenzymes in normal and hypertrophied human ventricular myocardium. Circ Res 1983;53:52-62.

[11] Nagai R, Pritzl N, Low RB, Stirewalt WS, Zak R, Alpert NR et al. Myosin isozyme synthesis and mRNA levels in pressure-overloaded rabbit hearts. Circ Res 1987;60:692-9.

[12] Alpert NR, Brosseau C, Federico A, Krenz M, Robbins J, Warshaw DM. Molecular mechanics of mouse cardiac myosin isoforms. Am J Physiol Heart Circ Physiol 2002;283:1446-54.

[13] Pope B, Hoh JF, Weeds A. The ATPase activities of rat cardiac myosin isoenzymes. FEBS Lett 1980;118:205-8.

[14] Harris DE, Work SS, Wright RK, Alpert NR, Warshaw DM. Smooth, cardiac and skeletal muscle myosin force and motion generation assessed by cross-bridge mechanical interactions in vitro. J Muscle Res Cell Motil 1994;15:11-9.

[15] Maughan DW. Kinetics and energetics of the crossbridge cycle. Heart Fail Rev 2005;10:175-85.

[16] Takashima S. Phosphorylation of Myosin regulatory light chain by Myosin light chain kinase, and muscle contraction. Circ J 2009;73:208-13.

[17] Bers DM, Guo T. Calcium signaling in cardiac ventricular myocytes. Ann N Y Acad Sci 2005;1047:86-98.

[18] Bers DM. Altered cardiac myocyte Ca regulation in heart failure. Physiology (Bethesda) 2006;21:380-7.

[19] Wu X, Bers DM. Sarcoplasmic reticulum and nuclear envelope are one highly interconnected Ca2+ store throughout cardiac myocyte. Circ Res 2006;99:283-91.

[20] Morano I. Tuning the human heart molecular motors by myosin light chains. Journal of Molecular Medicine 1999;77:544-55.

[21] Morano I. Effects of different expression and posttranslational modifications of myosin light chains on contractility of skinned human cardiac fibers. Basic Res Cardiol 1992;87 Suppl 1:129-41.

[22] Morano I, Hadicke K, Haase H, Bohm M, Erdmann E, Schaub MC. Changes in essential myosin light chain isoform expression provide a molecular basis for isometric force regulation in the failing human heart. J Mol Cell Cardiol 1997;29:1177-87.

[23] Westwood SA, Perry SV. The effect of adrenaline on the phosphorylation of the P light chain of myosin and troponin I in the perfused rabbit heart. Biochem J 1981;197:185-93.

[24] Moncrief ND, Kretsinger RH, Goodman M. Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. J Mol Evol 1990;30:522-62.

[25] White MY, Cordwell SJ, McCarron HC, Tchen AS, Hambly BD, Jeremy RW. Modifications of myosin-regulatory light chain correlate with function of stunned myocardium. J Mol Cell Cardiol 2003;35:833-40.

[26] Irving TC, Konhilas J, Perry D, Fischetti R, de Tombe PP. Myofilament lattice spacing as a function of sarcomere length in isolated rat myocardium. Am J Physiol Heart Circ Physiol 2000;279:2568-73.

[27] Kawai M, Wray JS, Zhao Y. The effect of lattice spacing change on cross-bridge kinetics in chemically skinned rabbit psoas muscle fibers. I. Proportionality between the lattice spacing and the fiber width. Biophys J 1993;64:187-96.

[28] Martyn DA, Gordon AM. Length and myofilament spacing-dependent changes in calcium sensitivity of skeletal fibres: effects of pH and ionic strength. J Muscle Res Cell Motil 1988;9:428-45.

[29] Levine RJ, Kensler RW, Yang Z, Stull JT, Sweeney HL. Myosin light chain phosphorylation affects the structure of rabbit skeletal muscle thick filaments. Biophys J 1996;71:898-907.

[30] Levine RJ, Chantler PD, Kensler RW, Woodhead JL. Effects of phosphorylation by myosin light chain kinase on the structure of Limulus thick filaments. J Cell Biol 1991;113:563-72.

[31] Metzger JM, Greaser ML, Moss RL. Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. Implications for twitch potentiation in intact muscle. J Gen Physiol 1989;93:855-83.

[32] Persechini A, Stull JT, Cooke R. The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. J Biol Chem 1985;260:7951-4.

[33] Sweeney HL, Stull JT. Phosphorylation of myosin in permeabilized mammalian cardiac and skeletal muscle cells. Am J Physiol 1986;250:657-60.

[34] Andersen GO, Qvigstad E, Schiander I, Aass H, Osnes JB, Skomedal T. Alpha(1)-AR-induced positive inotropic response in heart is dependent on myosin light chain phosphorylation. Am J Physiol Heart Circ Physiol 2002;283:1471-80.

[35] Rossmanith GH, Hoh JF, Turnbull L, Ludowyke RI. Mechanism of action of endothelin in rat cardiac muscle: cross-bridge kinetics and myosin light chain phosphorylation. J Physiol 1997;505:217-27.

[36] Riise J, Nguyen CH, Qvigstad E, Sandnes DL, Osnes JB, Skomedal T et al. Prostanoid F receptors elicit an inotropic effect in rat left ventricle by enhancing myosin light chain phosphorylation. Cardiovasc Res 2008;80:407-15.

[37] Qvigstad E, Sjaastad I, Brattelid T, Nunn C, Swift F, Birkeland JA et al. Dual serotonergic regulation of ventricular contractile force through 5-HT2A and 5-HT4 receptors induced in the acute failing heart. Circ Res 2005;97:268-76.

[38] Hussain RI, Qvigstad E, Birkeland JA, Eikemo H, Glende A, Sjaastad I et al. Activation of muscarinic receptors elicits inotropic responses in ventricular muscle from rats with heart failure through myosin light chain phosphorylation. Br J Pharmacol 2009;156:575-86.

[39] Somlyo AP, Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 2003;83:1325-58.

[40] Davis JS, Hassanzadeh S, Winitsky S, Lin H, Satorius C, Vemuri R et al. The overall pattern of cardiac contraction depends on a spatial gradient of myosin regulatory light chain phosphorylation. Cell 2001;107:631-41.

[41] Aoki H, Sadoshima J, Izumo S. Myosin light chain kinase mediates sarcomere organization during cardiac hypertrophy in vitro. Nat Med 2000;6:183-8.

[42] Seguchi O, Takashima S, Yamazaki S, Asakura M, Asano Y, Shintani Y et al. A cardiac myosin light chain kinase regulates sarcomere assembly in the vertebrate heart. J Clin Invest 2007;117:2812-24.

[43] Scruggs SB, Hinken AC, Thawornkaiwong A, Robbins J, Walker LA, de Tombe PP et al. Ablation of ventricular myosin regulatory light chain phosphorylation in mice causes cardiac dysfunction in situ and affects neighboring myofilament protein phosphorylation. J Biol Chem 2009;284:5097-106.

[44] Sanbe A, Fewell JG, Gulick J, Osinska H, Lorenz J, Hall DG et al. Abnormal cardiac structure and function in mice expressing nonphosphorylatable cardiac regulatory myosin light chain 2. Journal of Biological Chemistry 1999;274:21085-94.

[45] Poetter K, Jiang H, Hassanzadeh S, Master SR, Chang A, Dalakas MC et al. Mutations in either the essential or regulatory light chains of myosin are associated with a rare myopathy in human heart and skeletal muscle. Nat Genet 1996;13:63-9.

[46] Kerrick WG, Kazmierczak K, Xu Y, Wang Y, Szczesna-Cordary D. Malignant familial hypertrophic cardiomyopathy D166V mutation in the ventricular myosin regulatory light chain causes profound effects in skinned and intact papillary muscle fibers from transgenic mice. FASEB J 2009;23:855-65.

[47] Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer 2009;9:28-39.

[48] Moorhead GB, De W, V, Templeton G, Kerk D. Evolution of protein phosphatases in plants and animals. Biochem J 2009;417:401-9.

[49] Ruegg UT, Burgess GM. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol Sci 1989;10:218-20.

[50] Cohen P. Protein kinases--the major drug targets of the twenty-first century? Nat Rev Drug Discov 2002;1:309-15.

[51] Chan JY, Takeda M, Briggs LE, Graham ML, Lu JT, Horikoshi N et al. Identification of Cardiac-Specific Myosin Light Chain Kinase. Circ Res 2008;102:571-80.

[52] Birukov KG, Schavocky JP, Shirinsky VP, Chibalina MV, Van Eldik LJ, Watterson DM. Organization of the genetic locus for chicken myosin light chain kinase is complex: multiple proteins are encoded and exhibit differential expression and localization. J Cell Biochem 1998;70:402-13.

[53] Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. J Biol Chem 2001;276:4527-30.

[54] Herring BP, Dixon S, Gallagher PJ. Smooth muscle myosin light chain kinase expression in cardiac and skeletal muscle. Am J Physiol Cell Physiol 2000;279:1656-64.

[55] Zhi G, Ryder JW, Huang J, Ding P, Chen Y, Zhao Y et al. Myosin light chain kinase and myosin phosphorylation effect frequency-dependent potentiation of skeletal muscle contraction. Proc Natl Acad Sci U S A 2005;102:17519-24.

[56] Ohlmann P, Tesse A, Loichot C, Ralay RH, Roul G, Philippe C et al. Deletion of MLCK210 induces subtle changes in vascular reactivity but does not affect cardiac function. Am J Physiol Heart Circ Physiol 2005;289:2342-9.

[57] Ishikawa Y, Kurotani R. Cardiac myosin light chain kinase: a new player in the regulation of myosin light chain in the heart. Circ Res 2008;102:516-8.

[58] Barford D, Das AK, Egloff MP. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct 1998;27:133-64.

[59] Lesage B, Beullens M, Pedelini L, Garcia-Gimeno MA, Waelkens E, Sanz P et al. A complex of catalytically inactive protein phosphatase-1 sandwiched between Sds22 and inhibitor-3. Biochemistry 2007;46:8909-19.

[60] Eto M, Elliott E, Prickett TD, Brautigan DL. Inhibitor-2 regulates protein phosphatase-1 complexed with NimA-related kinase to induce centrosome separation. J Biol Chem 2002;277:44013-20.

[61] McCright B, Rivers AM, Audlin S, Virshup DM. The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J Biol Chem 1996;271:22081-9.

[62] Strack S, Zaucha JA, Ebner FF, Colbran RJ, Wadzinski BE. Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. J Comp Neurol 1998;392:515-27.

[63] Hirano K, Hirano M, Kanaide H. Regulation of myosin phosphorylation and myofilament Ca2+ sensitivity in vascular smooth muscle. J Smooth Muscle Res 2004;40:219-36.

[64] Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. Mol Cell 2009;33:537-45.

[65] Hurley TD, Yang J, Zhang L, Goodwin KD, Zou Q, Cortese M et al. Structural basis for regulation of protein phosphatase 1 by inhibitor-2. J Biol Chem 2007;282:28874-83.

[66] Takai A, Bialojan C, Troschka M, Ruegg JC. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett 1987;217:81-4.

[67] Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 1995;80:225-36.

[68] Mumby MC, Walter G. Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Physiol Rev 1993;73:673-99.

[69] Barford D, Das AK, Egloff MP. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct 1998;27:133-64.

[70] MacKintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett 1990;264:187-92.

[71] Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 1998;67:821-55.

[72] Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y et al. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun 1989;159:871-7.

[73] Kato Y, Fusetani N, Matsunaga S, Hashimoto K. Calyculins, potent antitumour metabolites from the marine sponge Discodermia calyx: biological activities. Drugs Exp Clin Res 1988;14:723-8.

[74] Kita A, Matsunaga S, Takai A, Kataiwa H, Wakimoto T, Fusetani N et al. Crystal structure of the complex between calyculin A and the catalytic subunit of protein phosphatase 1. Structure 2002;10:715-24.

[75] Ito M, Nakano T, Erdodi F, Hartshorne DJ. Myosin phosphatase: structure, regulation and function. Mol Cell Biochem 2004;259:197-209.

[76] Fujioka M, Takahashi N, Odai H, Araki S, Ichikawa K, Feng J et al. A new isoform of human myosin phosphatase targeting/regulatory subunit (MYPT2): cDNA cloning, tissue expression, and chromosomal mapping. Genomics 1998;49:59-68.

[77] Okamoto R, Kato T, Mizoguchi A, Takahashi N, Nakakuki T, Mizutani H et al. Characterization and function of MYPT2, a target subunit of myosin phosphatase in heart. Cell Signal 2006;18:1408-16.

[78] Bockaert J, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J 1999;18:1723-9.

[79] Oldham WM, Hamm HE. How do receptors activate G proteins? Adv Protein Chem 2007;74:67-93.

[80] Worzfeld T, Wettschureck N, Offermanns S. G(12)/G(13)-mediated signalling in mammalian physiology and disease. Trends Pharmacol Sci 2008;29:582-9.

[81] Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T et al. Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. Circ Res 1999;84:43-52.

[82] Dhein S, van Koppen CJ, Brodde OE. Muscarinic receptors in the mammalian heart. Pharmacol Res 2001;44:161-82.

[83] Totlandsdal AI, Skomedal T, Lag M, Osnes JB, Refsnes M. Pro-inflammatory potential of ultrafine particles in mono- and co-cultures of primary cardiac cells. Toxicology 2008;247:23-32.

[84] Skomedal T, Osnes JB, Oye I. Differences between alpha-adrenergic and beta-adrenergic inotropic effects in rat heart papillary muscles. Acta Pharmacol Toxicol (Copenh) 1982;50:1-12.

[85] Eikemo H, Skomedal T, Levy FO, Osnes JB. Calculating reaction rate constants and estimating the efficacy of selective enzyme inhibitors. Anal Biochem 2008;383:323-5.

[86] Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J 2003;371:199-204.

[87] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95-105.

[88] Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem 1993;268:9194-7.

[89] Saitoh M, Ishikawa T, Matsushima S, Naka M, Hidaka H. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. J Biol Chem 1987;262:7796-801.

[90] Saitoh M, Naka M, Hidaka H. The modulatory role of myosin light chain phosphorylation in human platelet activation. Biochem Biophys Res Commun 1986;140:280-7.

[91] Meggio F, Donella DA, Ruzzene M, Brunati AM, Cesaro L, Guerra B et al. Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. Eur J Biochem 1995;234:317-22.

[92] Meyer T, Regenass U, Fabbro D, Alteri E, Rosel J, Muller M et al. A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. Int J Cancer 1989;43:851-6.

[93] Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F. Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. Biochem Biophys Res Commun 1986;135:397-402.

[94] Gschwendt M, Kittstein W, Marks F. Elongation factor-2 kinase: effective inhibition by the novel protein kinase inhibitor rottlerin and relative insensitivity towards staurosporine. FEBS Lett 1994;338:85-8.

[95] Yanagihara N, Tachikawa E, Izumi F, Yasugawa S, Yamamoto H, Miyamoto E. Staurosporine: an effective inhibitor for Ca2+/calmodulin-dependent protein kinase II. J Neurochem 1991;56:294-8.

[96] Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 1997;389:990-4.

[97] Asano M. Divergent pharmacological effects of three calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine and calmidazolium, on isometric tension development and myosin light chain

phosphorylation in intact bovine tracheal smooth muscle. J Pharmacol Exp Ther 1989;251:764-73.

[98] Rokolya A, Singer HA. Inhibition of CaM kinase II activation and force maintenance by KN-93 in arterial smooth muscle. Am J Physiol Cell Physiol 2000;278:537-45.

[99] Hashimoto Y, Soderling TR. Phosphorylation of smooth muscle myosin light chain kinase by Ca2+/calmodulin-dependent protein kinase II: comparative study of the phosphorylation sites. Arch Biochem Biophys 1990;278:41-5.

[100] Ikebe M, Reardon S. Phosphorylation of smooth myosin light chain kinase by smooth muscle Ca2+/calmodulin-dependent multifunctional protein kinase. J Biol Chem 1990;265:8975-8.

[101] Tansey MG, Word RA, Hidaka H, Singer HA, Schworer CM, Kamm KE et al. Phosphorylation of myosin light chain kinase by the multifunctional calmodulin-dependent protein kinase II in smooth muscle cells. J Biol Chem 1992;267:12511-6.

[102] Tansey MG, Luby-Phelps K, Kamm KE, Stull JT. Ca(2+)-dependent phosphorylation of myosin light chain kinase decreases the Ca2+ sensitivity of light chain phosphorylation within smooth muscle cells. J Biol Chem 1994;269:9912-20.

[103] Driska SP, Aksoy MO, Murphy RA. Myosin light chain phosphorylation associated with contraction in arterial smooth muscle. Am J Physiol 1981;240:222-33.

[104] Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca2+-independent smooth muscle contraction. a novel function for integrin-linked kinase. J Biol Chem 2001;276:16365-73.

[105] Niiro N, Ikebe M. Zipper-interacting protein kinase induces Ca(2+)-free smooth muscle contraction via myosin light chain phosphorylation. J Biol Chem 2001;276:29567-74.

[106] Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T et al. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 1996;271:20246-9.

[107] Van Eyk JE, Arrell DK, Foster DB, Strauss JD, Heinonen TY, Furmaniak-Kazmierczak E et al. Different molecular mechanisms for Rho family GTPase-dependent, Ca2+-independent contraction of smooth muscle. J Biol Chem 1998;273:23433-9.

[108] Edelman AM, Lin WH, Osterhout DJ, Bennett MK, Kennedy MB, Krebs EG. Phosphorylation of smooth muscle myosin by type II Ca2+/calmodulin-dependent protein kinase. Mol Cell Biochem 1990;97:87-98.

[109] Chew TL, Masaracchia RA, Goeckeler ZM, Wysolmerski RB. Phosphorylation of non-muscle myosin II regulatory light chain by p21-activated kinase (gamma-PAK). J Muscle Res Cell Motil 1998;19:839-54.

[110] Komatsu S, Hosoya H. Phosphorylation by MAPKAP kinase 2 activates Mg(2+)-ATPase activity of myosin II. Biochem Biophys Res Commun 1996;223:741-5.

[111] Suizu F, Ueda K, Iwasaki T, Murata-Hori M, Hosoya H. Activation of actin-activated MgATPase activity of myosin II by phosphorylation with MAPK-activated protein kinase-1b (RSK-2). J Biochem 2000;128:435-40.

[112] Murata-Hori M, Fumoto K, Fukuta Y, Iwasaki T, Kikuchi A, Tatsuka M et al. Myosin II regulatory light chain as a novel substrate for AIM-1, an aurora/Ipl1p-related kinase from rat. J Biochem 2000;128:903-7.

[113] Alessi DR. The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1beta (Rsk-2) and p70 S6 kinase. FEBS Lett 1997;402:121-3.

[114] Roberts NA, Haworth RS, Avkiran M. Effects of bisindolylmaleimide PKC inhibitors on p90RSK activity in vitro and in adult ventricular myocytes. Br J Pharmacol 2005;145:477-89.

[115] Wilson DP, Sutherland C, Borman MA, Deng JT, Macdonald JA, Walsh MP. Integrin-linked kinase is responsible for Ca2+-independent myosin diphosphorylation and contraction of vascular smooth muscle. Biochem J 2005;392:641-8.

[116] Borman MA, Macdonald JA, Haystead TA. Staurosporine inhibition of zipper-interacting protein kinase contractile effects in gastrointestinal smooth muscle. Biochem Cell Biol 2007;85:111-20.

[117] Yu JS, Chang SH, Chan WH, Chen HC. Enzyme-linked immunosorbent assay for the determination of p21-activated kinase activity. J Biochem 2001;129:243-51.

[118] Rajashree R, Blunt BC, Hofmann PA. Modulation of myosin phosphatase targeting subunit and protein phosphatase 1 in the heart. Am J Physiol Heart Circ Physiol 2005;289:1736-43.

[119] Goeckeler ZM, Masaracchia RA, Zeng Q, Chew TL, Gallagher P, Wysolmerski RB. Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2. J Biol Chem 2000;275:18366-74.

[120] Frearson N, Perry SV. Phosphorylation of the light-chain components of myosin from cardiac and red skeletal muscles. Biochem J 1975;151:99-107.

[121] Morano I, Hofmann F, Zimmer M, Ruegg JC. The influence of P-light chain phosphorylation by myosin light chain kinase on the calcium sensitivity of chemically skinned heart fibres. FEBS Lett 1985;189:221-4.

[122] Morano I, Bachle-Stolz C, Katus A, Ruegg JC. Increased calcium sensitivity of chemically skinned human atria by myosin light chain kinase. Basic Res Cardiol 1988;83:350-9.

[123] Grimm M, Haas P, Willipinski-Stapelfeldt B, Zimmermann WH, Rau T, Pantel K et al. Key role of myosin light chain (MLC) kinase-mediated MLC2a phosphorylation in the alpha 1-adrenergic positive inotropic effect in human atrium. Cardiovasc Res 2005;65:211-20.

[124] Fitzsimons DP, Bodell PW, Baldwin KM. Phosphorylation of rodent cardiac myosin light chain 2: effects of exercise. J Appl Physiol 1989;67:2447-53.

[125] van d, V, Papp Z, Boontje NM, Zaremba R, de Jong JW, Janssen PM et al. The effect of myosin light chain 2 dephosphorylation on Ca2+ -sensitivity of force is enhanced in failing human hearts. Cardiovasc Res 2003;57:505-14.

[126] Du XY, Schoemaker RG, Bos E, Saxena PR. Different pharmacological responses of atrium and ventricle: studies with human cardiac tissue. Eur J Pharmacol 1994;259:173-80.

[127] Korth M, Kuhlkamp V. Muscarinic receptors mediate negative and positive inotropic effects in mammalian ventricular myocardium: differentiation by agonists. Br J Pharmacol 1987;90:81-90.

[128] Eglen RM, Montgomery WW, Whiting RL. Negative and positive inotropic responses to muscarinic agonists in guinea pig and rat atria in vitro. J Pharmacol Exp Ther 1988;247:911-7.

[129] Nouchi H, Kaeriyama S, Muramatsu A, Sato M, Hirose K, Shimizu N et al. Muscarinic receptor subtypes mediating positive and negative inotropy in the developing chick ventricle. J Pharmacol Sci 2007;103:75-82.

[130] Protas LL. Muscarinic receptors mediating the positive inotropic effect of carbachol in embryonic chick ventricle have a low affinity for pirenzepine. Eur J Pharmacol 1990;175:215-8.

[131] Imai S, Ohta H. Positive inotropic effects induced by carbachol in rat atria treated with islet-activating protein (IAP)--association with phosphatidylinositol breakdown. Br J Pharmacol 1988;94:347-54.

[132] McIvor ME, Orchard CH, Lakatta EG. Dissociation of changes in apparent myofibrillar Ca2+ sensitivity and twitch relaxation induced by adrenergic and cholinergic stimulation in isolated ferret cardiac muscle. J Gen Physiol 1988;92:509-29.

[133] Puceat M, Clement O, Lechene P, Pelosin JM, Ventura-Clapier R, Vassort G. Neurohormonal control of calcium sensitivity of myofilaments in rat single heart cells. Circ Res 1990;67:517-24.

[134] Skomedal T, Borthne K, Aass H, Geiran O, Osnes JB. Comparison between alpha-1 adrenoceptor-mediated and beta adrenoceptor-mediated inotropic components elicited by norepinephrine in failing human ventricular muscle. J Pharmacol Exp Ther 1997;280:721-9.

[135] Lutz S, Shankaranarayanan A, Coco C, Ridilla M, Nance MR, Vettel C et al. Structure of Galphaq-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science 2007;318:1923-7.

[136] Vogt A, Lutz S, Rumenapp U, Han L, Jakobs KH, Schmidt M et al. Regulator of G-protein signalling 3 redirects prototypical Gi-coupled receptors from Rac1 to RhoA activation. Cell Signal 2007;19:1229-37.